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The biology of peroxisomes in *Hansenula polymorpha*

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Summary

Archaeobacteria, eubacteria and eukaryotes form the three kingdoms of life. The architecture of the archae- and eubacteria is relatively simple, consisting of cytoplasm (containing the genetic information [DNA], proteins and chemical components e.g. intermediates of specific reactions in the cell) that is surrounded by a lipid bi-layer membrane and a protective cell wall. Eukaryotes are more complex of architecture. Eukaryote cells are characterized by the presence of membrane enclosed compartments that are called organelles. These organelles are the sites of specific enzymatic reactions and are separated from the cytosol because they may be either harmful for the cell or require specific conditions for optimal reaction.

A well known organelle is the nucleus that is known for storing the DNA and to which eukaryotes owe their name (eu karyos; with good or true nucleus). Other important organelles are the endoplasmatic reticulum (e.g. involved in the synthesis and folding of proteins destined for export out of the cell), the vacuole (which can be considered “waste basket” that stores and recycles redundant cellular material) and mitochondria (the energy generators of the cell).

A less familiar class of organelles are the microbodies. Microbodies are discovered by Rhodin (1956) and first biochemically characterized in the 1960s by Christian de Duve. Four sub-classes of microbodies are recognized namely *i*) peroxisomes, *ii*) glyoxysomes, *iii*) glycosomes and *iv*) hydrogenosomes, based on their major metabolic function. Microbodies are ubiquitous organelles but were regarded not very important until it became apparent that peroxisomes were linked to several severe human inherited diseases, some of which are lethal. The variance in metabolic functions of microbodies is unprecedented that can range from a function in photorespiration in plants to etherlipid synthesis in mammals until the production of secondary metabolites e.g. β -lactam antibiotics in filamentous fungi.

Microbodies do not contain their own DNA. The proteins required for the development of a functional peroxisome (peroxisome biogenesis) are conserved between all eukaryotes. These proteins are called peroxins (abbreviated as Pex). Functions and sequence

information of peroxins in lower eukaryotes are highly similar in higher eukaryotes, and as such this information was successfully used understanding the molecular details of the mutations in peroxins that led to human peroxisome biogenesis disorders.

Cells generally contain multiple peroxisomes, their actual number being dependent on environmental conditions. A typical example of peroxisome number controlled by growth conditions is observed in the methylotrophic yeast *Hansenula polymorpha*. These cells are capable to grow on methanol as sole carbon and energy source, the metabolism of which requires the peroxisomal enzymes alcohol oxidase (AO), catalase (CAT) and dihydroxyacetone synthase (DHAS).

Consequently, peroxisomes massively develop during growth of cells on methanol and may occupy up to ~50% of the total cellular volume. If these cells are shifted to fresh cultivation media in which peroxisomes are considered redundant for growth (e.g. excess glucose), the peroxisomes present in the cells are rapidly and sequentially degraded inside the vacuole. This glucose-inducible degradation process is termed macropexophagy.

The main topic of this thesis is a detailed analysis of a component of the peroxisomal membrane, Pex14p, that is important for peroxisome biogenesis as well as for macropexophagy. Chapter 1 summarizes the current knowledge on the function of Pex14 and the interactions this protein has with other peroxins and will furthermore address the role of SNARE proteins in peroxisome biogenesis and macropexophagy.

We distinguish between two types of peroxisomes, mature peroxisomes, which are in essence enzyme bags and young, nascent peroxisomes that actively grow by import of matrix proteins. Mature peroxisomes most likely contain less amounts of Pex14p than nascent ones. This raises the question whether the amounts of Pex14p that are required for biogenesis equal those essential for macropexophagy. Chapter 2 addresses this question and shows that only catalytical amounts of Pex14p are sufficient to promote macropexophagy but can not support normal organelle biogenesis. This has been accomplished by using the promoter of the gene encoding for the lowest expressed peroxin known, namely *PEX4*. Peroxisome degradation via nitrogen limitation induced microautophagy is not disturbed even in the absence of Pex14p. Another observation was that during growth on methanol, Pex14p occurs in two forms, normal Pex14p and modified by phosphorylation, Pex14p^{Pi}. We demonstrate that Pex14p^{Pi} is the first to disappear during

macropexophagy. In a time span of 30 minutes after induction of macropexophagy most of the Pex14p^{Pi} has disappeared.

Peroxisome biogenesis is severely disturbed at the reduced Pex14p levels a phenomenon that was associated with mislocalization of AO, CAT and DHAS in the cytosol. We concluded that Pex14p acts as a switch determining the fate of peroxisomes by discriminating between organelle development and turnover

Peroxisomal matrix proteins contain a specific targeting signal, the so-called **peroxisomal targeting signal (PTS)**. Proteins carrying these signals are sorted to the peroxisome docking complex, consisting of the proteins Pex13p, Pex14p and Pex17p. Two PTSs have been defined, namely a C-terminal PTS1 signal (SKL-COOH) and an N-terminal (located within the first ~50 amino acids) PTS2 signal (R-(L/V/I)-XX-(L/V/I/H)-(L/S/G/A)-X-(H/Q)-(L/A)). Pex5p is a soluble protein that is identified as the receptor for PTS1 proteins. Similarly, Pex7p forms the receptor for PTS2 proteins. Pex5p and Pex7p transport their cargo to peroxisomes via binding with the docking complex at the peroxisome membrane. Pex14p recognizes specific sequences in the peroxins Pex5p and Pex7p. In chapter 3 we investigated the N-terminus of Pex14p and its importance in recognition of (the receptor proteins) Pex5p and Pex7p. The N-terminus of Pex14p is highly conserved among all eukaryotes. Low resolution structural analysis reveals that the N-terminus of *H. polymorpha* Pex14p adopts a predominantly alpha helical shape. In conjunction with the structural data, prediction software JPRED demonstrates three defined alpha-helical domains in the first 58 amino acids. The first alpha-helix was predicted between residues 10 and 21. A deletion of the first 9 amino acids had no effect on methanol utilization, nor import of green fluorescent protein tagged with a PTS1 sequence (GFP.SKL). However, a deletion of the first 21 amino acids resulted in a loss of function of Pex14p. To determine whether the sequence or the alpha-helical shape was important, amino acids from 10 to 21 were mutagenized to amino acids of opposing properties. An alanine to proline mutation at position 17 was introduced to break the alpha-helix. Only the helix breaking mutation and substitution of two other residues (F20S and L21D) resulted in a defect in PTS1 protein import. We concluded that the helical structure and both the phenylalanine and leucine in positions 20 and 21 respectively are important features for

recognizing and binding PTS1 receptor protein Pex5p. Surprisingly, the same residues are also required for PTS2 import. GFP, N-terminally fused to the PTS2 sequence of thiolase, did not localize to peroxisomes in these mutants. Hence, most likely the interaction of Pex14p with Pex7p (or Pex20p to which Pex7p does bind) is disturbed as well.

In chapter 4 we investigate the role of the protein Ypt7p in the biogenesis and degradation of peroxisomes. Ypt7p is known to function in the fusion of membranes of the vacuole (vacuole-vacuole fusions), but is also involved in fusion of autophagosomes (structures that contain cargo for degradation in the vacuole) with the vacuole. Ypt7p is a Rab-GTPase that is essential for Vam7p to localize to the vacuolar membrane and become active. Vam7p is a cytosolic soluble NSF attachment receptor (SNARE) protein that is also essential for the above fusion process. However, Vam7p appeared to be involved in both peroxisome biogenesis and degradation. Similar to *vam7* cells, electron microscopical analysis of *ypt7* cells revealed that peroxisomes were enwrapped by multiple membrane layers. This aberrant peroxisome phenotype became apparent at a stage of growth at which the first proliferation (by fission) of the organelles took place. Notwithstanding the aberrant organelle development, *ypt7* cells grow normally on methanol like WT, indicating that the abnormal peroxisomal structure in *ypt7* cells did not affect the metabolic processes of the organelles. Furthermore, both macropexophagy and microautophagy was blocked in *ypt7* cells, like in *vam7* cells. In conclusion, this implies that the machinery normally associated with vacuolar fusion events, also functions in peroxisome biogenesis. Possibly, peroxisome biogenesis requires vesicle fusion processes.

As indicated above, the canonical PTS1 is the C-terminal sequence –SKL–COOH. However, not all proteins that are targeted to the peroxisome via a PTS1 have –SKL–COOH as a targeting signal. [ACS]–[KR]–[LM]–COOH was postulated as a consensus that may describe most of the PTS1 proteins, based on proteins found in mammals and baker's yeast. However, the key methanol metabolism enzymes of both *H. polymorpha* and *Pichia pastoris* contain PTS1 sequences that do not fit this consensus, AO has an –ARF–COOH in both organisms, and DHAS has an –NKL–COOH in *H. polymorpha* and a –DKL–COOH in *P. pastoris*. In chapter 5, we describe a method to identify putative novel PTS1 proteins

based on the assumption that proteins with the same function and high similarity in sequence have similar localizations in closely related organisms. This way sequence similarity can be used to predict whether or not a protein localizes to the peroxisome via a PTS1 in *H. polymorpha* and *P. pastoris*. If a protein has an a-typical PTS1 that does not fit the [ACS]-[KR]-[LM]-COOH sequence, but closely related proteins in other organisms do have this typical PTS1, we assume that this a-typical sequence may represent a bona fide PTS1 sequence in methylotrophic yeast. Using this method we describe the PTS1 of both organisms and identify 34 PTS1 proteins in *H. polymorpha* and 30 PTS1 proteins in *P. pastoris*. Proof of principle was presented by fusing GFP to the N-terminus of a putative acetylspermidine oxidase (-GKL-COOH) which localizes indeed to peroxisomes, even though the PTS1 of this protein is considered a-typical for a PTS1 sequence.